# Association of inhibitory tyrosine protein kinase p50$^{csk}$ with prot in tyrosine phosphatase PEP in T cells and other hemopoietic cells



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**p50$^{csk}$ is a tyrosine protein kinase (TPK) that represses the activity of Src family TPKs. We previously showed that Csk is a potent negative regulator of antigen receptor signaling in T lymphocytes and that its Src homology (SH) 3 and SH2 domains are required to inhibit these signals. To test the idea that the Csk SH3 and SH2 domains mediate interactions with other cellular proteins, we attempted to identify Csk-associated polypeptides using the yeast two-hybrid system. The results of our experiments demonstrated that Csk physically associates with PEP, a protein tyrosine phosphatase (PTP) expressed in hemopoietic cells. Further analyses revealed that this interaction was mediated by the Csk SH3 domain and by a proline-rich region (PPPLPERTP) in the non-catalytic C-terminal portion of PEP. The association between Csk and PEP was documented in transiently transfected Cos-1 cells and in a variety of cells of hemopoietic lineages, including T cells. Additional analyses demonstrated that the association between Csk and PEP is highly specific. Together, these data indicated that PEP may be an effector and/or a regulator of p50$^{csk}$ in T cells and other hemopoietic cells. Moreover, they allowed the identification of PEP as the first known ligand for the Csk SH3 domain.**

*Keywords:* Csk/PEP/protein tyrosine phosphatase/T cell activation/tyrosine protein kinase

## Introduction

Csk is a 50 kDa cytoplasmic tyrosine protein kinase (TPK) expressed in all cell types, albeit at higher levels in cells of neuronal and hemopoietic lineages (Nada *et al.*, 1991; Bergman *et al.*, 1992; Sabe *et al.*, 1992). Like Src family kinases, Csk bears N-terminal Src homology (SH) 3 and SH2 sequences, in addition to a C-terminal catalytic domain. In contrast to Src-related enzymes, however, Csk is devoid of N-terminal membrane targeting sequences, a site of autophosphorylation and an inhibitory C-terminal tyrosine. Considerable interest in the function and regulation of Csk stems from its ability to inactivate Src family kinases, through phosphorylation of their inhibitory C-terminal tyrosine (reviewed in Cooper and Howell, 1993). The importance of Csk in cellular physiology is exemplified by the observation that Csk-deficient mice, produced by homologous recombination in embryonic stem (ES) cells, exhibited marked developmental abnormalities in the central nervous system and early embryonic lethality (Imamoto and Soriano, 1993; Nada *et al.*, 1993). Furthermore, Csk-deficient ES cells failed to differentiate into mature T and B cells when injected into RAG-1-deficient blastocysts (Gross *et al.*, 1995).

The role of p50$^{csk}$ in mature cells was first revealed by the observation that overexpression of Csk in an antigen-specific T cell line (BI-141) caused a strong inhibition of antigen receptor-induced responses, including tyrosine protein phosphorylation and lymphokine secretion (Chow *et al.*, 1993). This inhibitory effect could be rescued by expression of Src family kinases bearing a mutation of the inhibitory C-terminal tyrosine, thereby being refractory to regulation by Csk (Chow *et al.*, 1993; L.M.L.Chow and A.Veillette, unpublished results). In a similar way, recent studies showed that enforced expression of p50$^{csk}$ inhibited the acid-induced $Na^+/H^+$ antiporter in kidney epithelial cells (Yamaji *et al.*, 1995) and endothelin-1-mediated responses in vascular cells (Simonson *et al.*, 1996).

Recently, several groups identified a second member of the Csk family, variably termed Ntk, Ctk, Matk, Lsk, Hyl, Batk and Bhk (reviewed in Chow and Veillette, 1995). As a result of an agreement between several of these laboratories, the Csk-related enzyme is now named Chk, for Csk homologous kinase. In contrast to Csk, Chk exclusively accumulates in brain and hemopoietic cells. While Chk also phosphorylates the inhibitory C-terminal tyrosine of Src family kinases *in vitro* and in yeast cells (Chow *et al.*, 1994a; Klages *et al.*, 1994), we recently observed that it is inefficient at negatively regulating antigen receptor-mediated signals in T cells (L.M.L.Chow and A.Veillette, unpublished results). Even though the exact basis for this difference is not known, it implies that the function and/or regulation of the two members of the Csk family are fundamentally distinct.

The regulation of p50$^{csk}$ is poorly understood. Previous studies have failed to provide convincing evidence that it undergoes phosphorylation *in vivo*. As the targets of Csk (i.e. Src family kinases) are located at the plasma membrane, it has been hypothesized that Csk is regulated by changes in cellular localization. In keeping with this idea, we demonstrated that constitutive membrane targeting of p50$^{csk}$ enhanced its ability to repress antigen receptor-mediated signals in T cells (Chow *et al.*, 1993). Even though the exact process by which Csk is physiologically recruited to cellular membranes has not been elucidated, accumulating data suggest that this function may be carried out by its SH3 and SH2 regions, which bind proline-rich motifs and tyrosine phosphorylated sequences respectively (Howell and Cooper, 1994; Bergman *et al.*, 1995; Cloutier *et al.*, 1995; reviewed in Pawson and Gish, 1992; Cohen *et al.*, 1995).

Further to their role in membrane recruitment, it is likely that the SH3 and SH2 domains of Csk provide additional functions. This view was suggested by our observation that constitutive membrane targeting at best partially relieved the requirement for the SH3 and SH2 domains for the ability of $p50^{csk}$ to repress T cell antigen receptor signaling (Cloutier *et al.*, 1995). Presumably, these sequences also interact with effectors and/or regulators that are necessary for the proper function of $p50^{csk}$. Even though little is known of the identity of these proteins, Sabe *et al.* (1994) reported that the Csk SH2 domain can associate with tyrosine phosphorylated paxillin and $p125^{fak}$. Similarly, Neet and Hunter (1995) showed that the GTPase activating protein (GAP)-associated p62 can bind the Csk SH2 domain in fibroblasts. Finally, we and others reported that the SH2 region of $p50^{csk}$ can bind several tyrosine phosphorylated proteins from activated T cells (Oetken *et al.*, 1994; Cloutier *et al.*, 1995).

To formally identify proteins binding the SH3 and/or SH2 domains of Csk in T cells, we have used the yeast two-hybrid system, which provides a sensitive strategy to identify protein–protein interactions (Fields and Song, 1989). Using Csk as a 'bait', we found that it can interact with the C-terminal half of PEP, a non-receptor PTP expressed in hemopoietic cells (Matthews *et al.*, 1992). This association is mediated by the Csk SH3 domain and by a proline-rich motif present in the C-terminal region of PEP. Further studies showed that Csk–PEP complexes are present in a variety of hemopoietic cell types, including T cells. By interacting with the SH3 region of $p50^{csk}$, PEP may be an important regulator and/or effector of Csk-mediated functions in T cells, as well as in other hemopoietic cells.

## Results

### *Identification of a putative Csk-associated protein using the yeast two-hybrid system*

A full-length *csk* cDNA (Nada *et al.*, 1991) was cloned in the yeast expression vector pBTM116. Following transformation of yeast strain L40, this construct caused expression of a lexA–Csk fusion protein, in which the DNA binding domain of lexA was fused to Csk (see Materials and methods). In an attempt to identify Csk binding proteins, a mouse splenocyte cDNA library cloned in the vector pVP16 was introduced into lexA–Csk-containing cells. Because pVP16 also contains the VP16 transactivation domain, the binding of library-encoded polypeptides to lexA–Csk conferred growth in histidine-deficient medium and expression of β-galactosidase activity. Ninety five histidine-independent colonies were chosen for further study. These colonies also exhibited β-galactosidase activity, a presumed consequence of the interaction of the Csk 'bait' with library-encoded sequences. To verify that the β-galactosidase activity resulted from interactions between library-encoded polypeptides and Csk, lexA–Csk was eliminated by growth in medium containing tryptophan and histidine. As a result, 90 colonies lost their β-galactosidase activity, implying that they represented 'true positives'. This was confirmed by showing that re-introduction of lexA–Csk, but not lexA alone or other lexA fusion proteins, restored β-galactosidase activity (data not shown). Plasmids were isolated from these colonies, transferred into *Escherichia coli*, and their inserts were analyzed by restriction digestion and/or sequencing. All 90 clones were found to contain an identical insert of 882 nt, in-frame with *lexA* (Figure 1A; data not shown).

Sequence analysis revealed that the putative Csk-associated sequence was PEP, a 120 kDa non-receptor PTP expressed in hemopoietic cells (Matthews *et al.*, 1992). Structurally, PEP contains an N-terminal catalytic domain and a long C-terminal region rich in prolines, glutamates, serines and threonines (Figure 1B). This last feature is reminiscent of 'PEST' sequences (Rogers *et al.*, 1986), which suggested that PEP might be a short lived polypeptide. However, a previous report demonstrated that the half-life of PEP is relatively long (>5 h) in mammalian cells (Flores *et al.*, 1994). As depicted in Figure 1B, the region identified by the yeast two-hybrid screen was located in the non-catalytic C-terminal domain of PEP.

### *The SH3 domain of Csk interacts with a proline-rich motif in the non-catalytic C-terminal portion of PEP*

To delineate the sequences implicated in the interaction between $p50^{csk}$ and PEP, Csk mutants lacking the SH3 (ΔSH3 Csk) or SH2 (ΔSH2 Csk) region or rendered kinase-defective by mutation of the ATP binding site [Lys222→Arg222 (R222 Csk)] were expressed with the C-terminal portion of PEP in the yeast two-hybrid system (Figure 2A). An anti-Csk immunoblot showed that all polypeptides were expressed in comparable amounts (data not shown). The ability of these various proteins to associate with PEP was determined by assaying yeast cells for β-galactosidase activity. While expression of wild-type Csk, ΔSH2 Csk and R222 Csk caused similar β-galactosidase activity, we observed that introduction of ΔSH3 Csk failed to stimulate this enzymatic activity (Figure 2A). To establish that the Csk SH3 sequence was sufficient for interacting with the C-terminal segment of PEP, the impact of expression of a lexA–Csk SH3 domain fusion was examined. This experiment revealed that the lexA–Csk SH3 domain fusion was sufficient to increase β-galactosidase activity, in a manner analogous to wild-type Csk (Figure 2A).

We also wanted to determine which PEP sequence(s) was involved in binding to Csk. To this end, a series of N-terminal and C-terminal truncations was created, using a C-terminal segment of PEP (C-PEP) as template (Figure 2B). C-PEP encompassed the sequence identified by the yeast two-hybrid system, in addition to the residues located C-terminal to this portion of PEP. The capacity of the truncated fragments to interact with wild-type Csk was ascertained as described above. Because SH3 domains bind proline-rich motifs (reviewed in Pawson and Gish, 1992; Cohen *et al.*, 1995), special attention was given to four proline-rich sequences present in the C-terminus of PEP (Figure 2B). ΔN1 C-PEP, which contained all four proline motifs, stimulated β-galactosidase activity to an extent comparable with C-PEP. However, ΔN2 C-PEP, missing P1, caused significantly reduced β-galactosidase activation, while ΔN3 C-PEP, which was devoid of both P1 and P2, but contained P3 and P4, was unable to stimulate any enzymatic activity. ΔC1 C-PEP and ΔC2 C-PEP, missing P3 and P4 and P2, P3 and P4 respectively, augmented β-galactosidase activity in a manner analogous

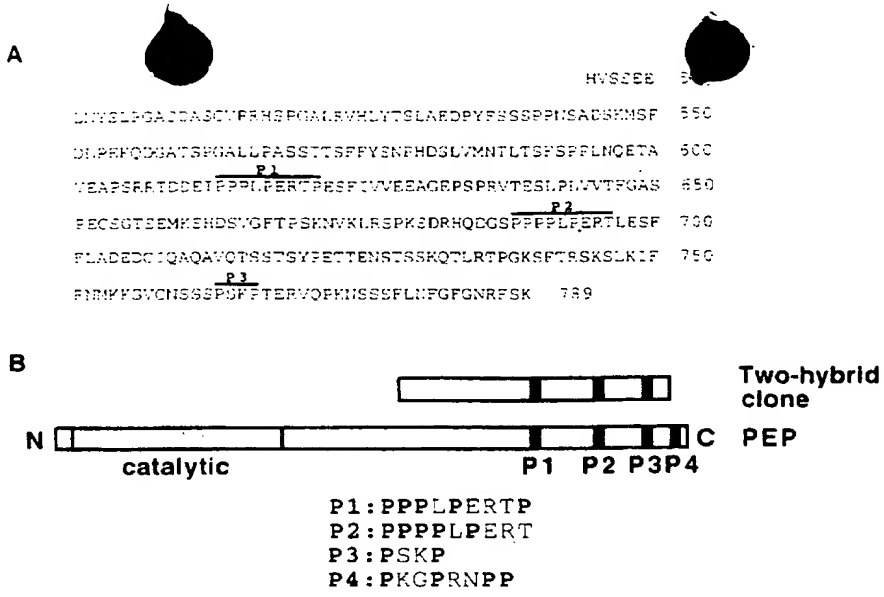


**Fig. 1.** Identification of PEP as a potential Csk binding protein using the yeast two-hybrid system. (**A**) Predicted polypeptide encoded by the cDNA clone isolated in the yeast two-hybrid screen. Amino acid numbering is based on the sequence of PEP (Matthews *et al.*, 1992). The positions of three proline-rich motifs are highlighted. (**B**) Schematic representation of PEP. The locations of the catalytic domain and of the proline-rich regions present in the non-catalytic C-terminal tail of PEP are indicated. The relative position of the sequence identified by the yeast two-hybrid screen is shown at the top.

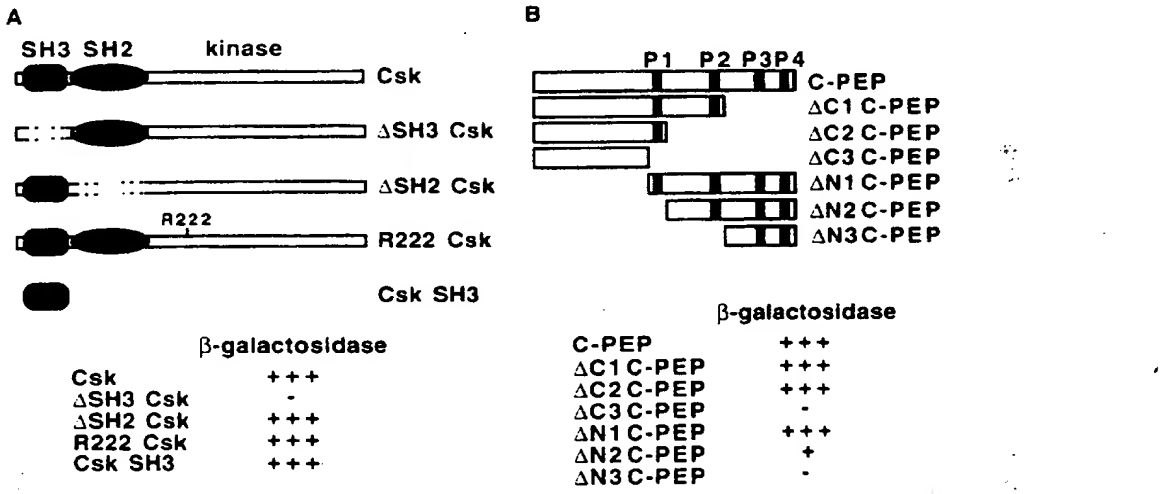


**Fig. 2.** Structural requirements for Csk–PEP interaction in the yeast two-hybrid system. (**A**) Csk sequences required for Csk–PEP interaction. The structures of the various Csk polypeptides co-expressed with the C-terminal fragment of PEP in yeast are shown at the top. Resulting induction of β-galactosidase activity is indicated at the bottom (–, no induction, even after 24 h incubation with the β-galactosidase substrate: +++, strong induction, visible within 15 min of addition of substrate). (**B**) PEP sequences necessary for Csk–PEP interaction. The structures of the various fragments of PEP which were studied are shown at the top. Resulting induction of β-galactosidase activity is indicated at the bottom (–, no induction, even after 24 h incubation with the β-galactosidase substrate: +, weak activation, noticeable after 24 h incubation with substrate: +++, strong induction, visible within 15 min of addition of substrate).

to C-PEP. In contrast, ΔC3 C-PEP, in which all four proline-rich domains were truncated, had no effect. In combination, these results indicate that in the yeast two-hybrid system, the SH3 domain of $p50^{csk}$ interacted with sequences encompassing the proline-rich regions P1 (PPPLPERTP) and, to a lesser extent, P2 (PPPPLPERT) of PEP.

In order to evaluate the specificity of the interaction between Csk and PEP, an *in vitro* binding assay was developed. Glutathione *S*-transferase (GST) fusion proteins containing the SH3 domain of Csk (GST–SH3 Csk) were produced in bacteria and purified on agarose–glutathione beads. These were incubated with bacterial lysates containing trpE fusion proteins bearing various fragments of PEP (Figure 3A). An anti-trpE immunoblot of total bacterial lysates showed that all trpE fusions were expressed in comparable amounts (Figure 3B). After several washes, bound proteins were resolved by SDS–PAGE and detected by immunoblotting with anti-trpE antibodies (Figure 3C). While GST–SH3 Csk did not react with trpE alone (lane 7), it clearly associated with a fusion protein including P1 of PEP (SRRTDDEI*PPPLPERTP*ES-FIVVEE; lane 10). In contrast, it did not bind trpE polypeptides encompassing P2 (SDRHQDGS*PPPPLP*-

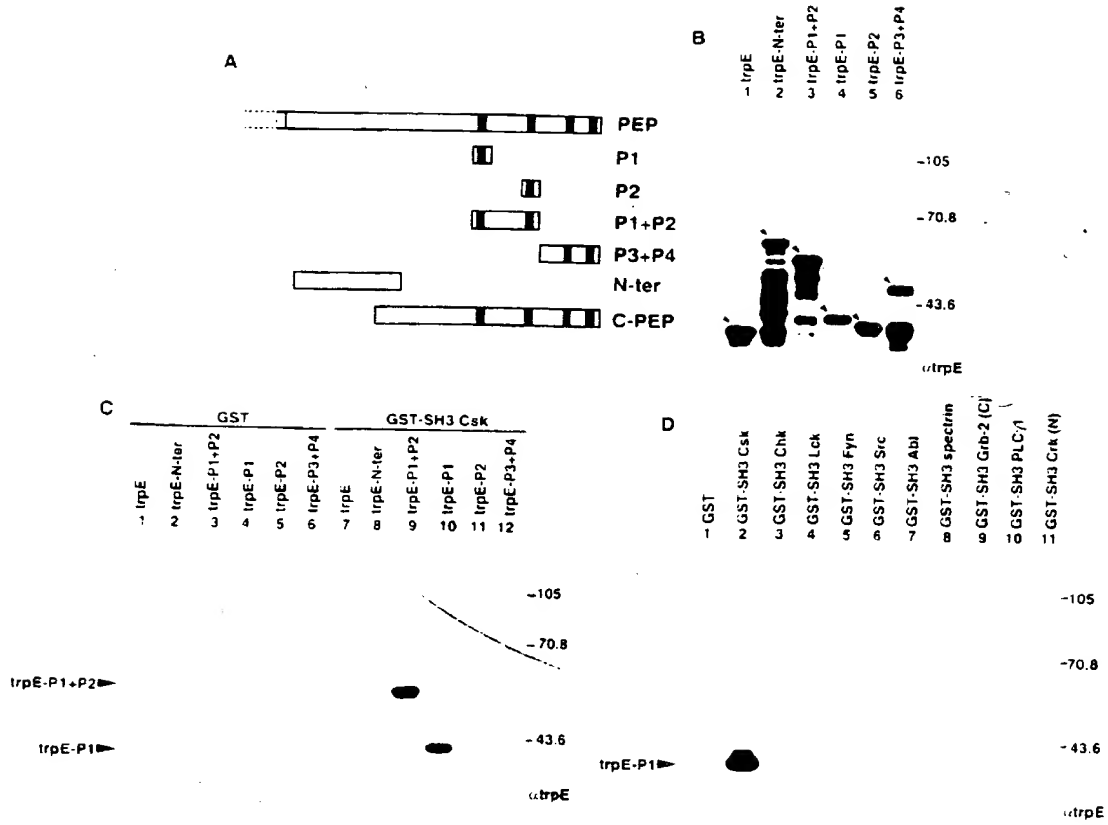


**Fig. 3.** Association of the SH3 domain of $p50^{csk}$ with PEP *in vitro*. (**A**) Fragments of PEP tested in these assays. All fragments were fused to trpE and produced in bacteria. (**B**) Expression of trpE–PEP fusion proteins in bacteria. Bacterial lysates were immunoblotted with anti-trpE antibodies. Migration of the fusion proteins is indicated by arrows, while that of molecular mass markers is shown on the right in kDa. Exposure time 5 h. (**C**) Interaction of the SH3 domain of Csk with various trpE–PEP fusion proteins. Bacterial lysates expressing the various trpE–PEP fusion proteins were incubated with GST or GST–Csk SH3 domains, previously immobilized on agarose–glutathione beads. After several washes, bound proteins were detected by immunoblotting with anti-trpE antibodies. Migration of the trpE–PEP fusion proteins is indicated on the left, while that of molecular mass markers is shown on the right in kDa. Exposure time 11 h. (**D**) Binding of trpE–P1 to other SH3 domains. The ability of trpE–P1 to associate with various GST–SH3 domains was ascertained as described for (C). Migration of trpE–P1 is indicated on the left, while the positions of molecular mass markers are shown on the right in kDa. Exposure time 12 h.

*ERT*LESFFLADE; lane 11), P3 and P4 (lane 12) or the N-terminal portion of the non-catalytic domain of PEP (lane 8).

The ability of P1 to associate with other SH3 domains *in vitro* was also examined (Figure 3D). Whereas trpE–P1 was stably bound to GST–Csk SH3 (lane 2), it failed to react with a GST fusion encompassing the SH3 domain of the Csk-related enzyme Chk (lane 3). Similarly, trpE–P1 did not associate with the SH3 region of $p56^{lck}$ (lane 4), $p59^{fyn}$ (lane 5), $p60^{src}$ (lane 6), Abl (lane 7), spectrin (lane 8), Grb-2 (lane 9), phospholipase C-γ1 (lane 10) or Crk (lane 11).

### *Reconstitution of the association between $p50^{csk}$ and PEP in Cos-1 cells*

To ensure that $p50^{csk}$ can interact with the full-length PEP protein in mammalian cells, transient transfection experiments were conducted in Cos-1 cells. To avoid interference from the endogenous Csk, three copies of an influenza hemagglutinin (HA)-derived sequence were fused to the C-terminus of Csk. This HA 'tag' could be detected with monoclonal antibody (mAb) 12CA5. In addition, to facilitate recognition of PEP, a 'tag' from the Myc protein was added to its N-terminus. This epitope reacted with mAb 9E10.

Sixty hours after transfection of *csk* and *pep* cDNAs, Cos-1 cells were lysed in NP-40-containing buffer and the association of Csk with PEP was determined by immunoblotting of anti-HA immunoprecipitates with anti-Myc mAb 9E10 (Figure 4A, top panel). Additionally, expression of the appropriate polypeptides was verified by immunoblotting of total cell lysates with either anti-HA (middle panel) or anti-Myc (lower panel) antibodies. In Cos-1 cells transfected with wild-type *csk* and *pep* cDNAs, significant amounts of PEP co-immunoprecipitated with $p50^{csk}$ (Figure 4A, lane 7). Similarly, PEP was associated with ΔSH2 Csk (lane 9) and R222 Csk (lane 10). In contrast, however, no PEP was bound to ΔSH3 Csk (lane 8). We also evaluated the ability to recover $p50^{csk}$ in immunoprecipitates of PEP. To this end, PEP was immunoprecipitated using a polyclonal rabbit antiserum directed against its non-catalytic portion and the presence of Csk was monitored by immunoblotting with anti-HA antibodies (Figure 4B). This study demonstrated that significant quantities of Csk were recovered in anti-PEP immunoprecipitates obtained from cells co-expressing PEP and Csk (lane 4), while no Csk was present in anti-PEP

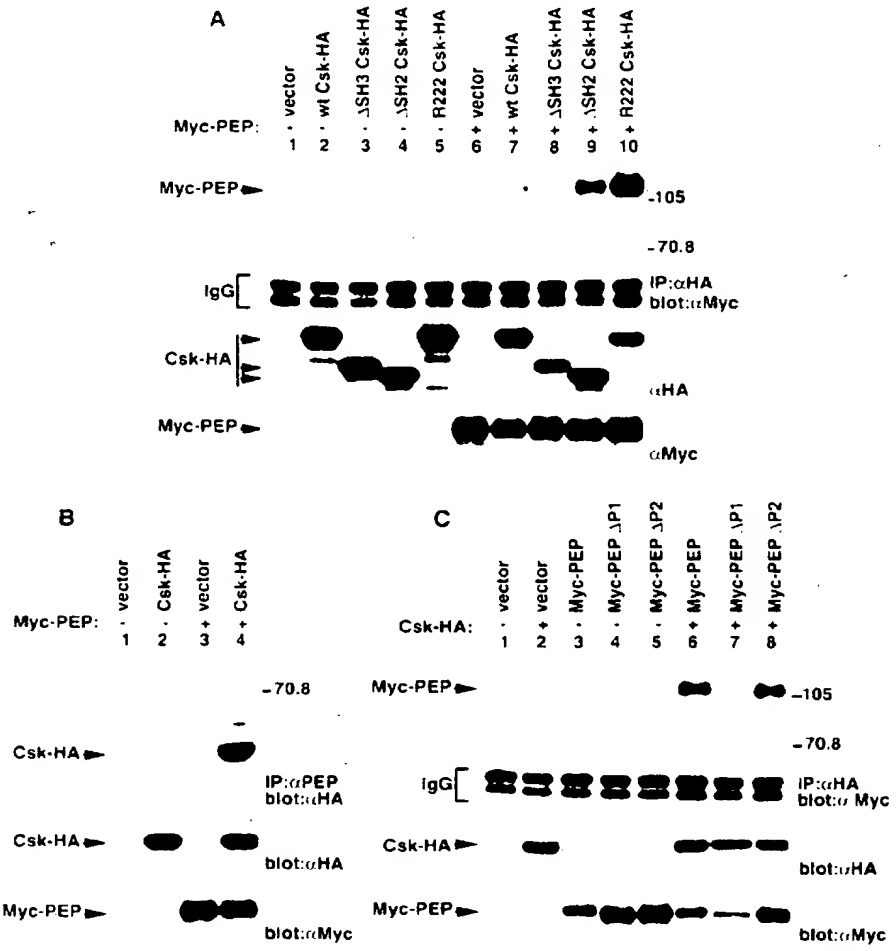


**Fig. 4.** Association of $p50^{csk}$ and PEP in Cos-1 cells. (A) The SH3 domain of Csk is necessary for binding to PEP in Cos-1 cells. Cos-1 cells were transfected with the indicated cDNAs, as outlined in Materials and methods. After 60 h, HA-tagged Csk polypeptides were recovered by immunoprecipitation with anti-HA mAb 12CA5 and the presence of Myc–PEP in these immunoprecipitates was determined by immunoblotting with anti-Myc mAb 9E10 (top panel). Expression of Csk–HA and Myc–PEP polypeptides was verified by immunoblotting of equivalent amounts of cell lysates with mAb 12CA5 (middle panel) and mAb 9E10 (bottom panel) respectively. The positions of Myc–PEP, the heavy chain of IgG and Csk–HA are indicated on the left; those of prestained molecular weight markers are shown on the right in kDa. Exposure time 24 h. (B) Detection of $p50^{csk}$ in anti-PEP immunoprecipitates. As outlined for (A), except that lysates were immunoprecipitated with rabbit anti-PEP polyclonal antibodies followed by immunoblotting with anti-HA mAb 12CA5. Migration of Csk–HA and Myc–PEP is indicated on the left, while that of prestained molecular weight markers is shown on the right in kDa. Exposure time 7 h. (C) The proline-rich region P1 of PEP is necessary for binding to $p50^{csk}$. As described for (A). Exposure times: top panel 24 h; middle panel 12 h; bottom panel 24 h. Migration of Myc–PEP, the heavy chain of IgG and Csk–HA is indicated on the left, while that of prestained molecular weight markers is shown on the right in kDa.

immunoprecipitates isolated from cells expressing $p50^{csk}$ alone (lane 2).

PEP mutants carrying deletions in the proline-rich regions P1 or P2 were also created and tested in these assays. As shown in Figure 4C, these experiments showed that the association of PEP with Csk was abolished by removal of the nine amino acids of P1 (PPPLPERTP; lane 7). In contrast, deletion of the nine amino acids of P2 (PPPPLPERT) had no effect (lane 8). In combination, these findings demonstrate that full-length Csk and PEP can interact in mammalian cells and that this association is mediated by the Csk SH3 domain and the first proline-rich region (P1) of PEP.

### *Detection of Csk–PEP complexes in T cells and other hemopoietic cells*

To ensure the physiological relevance of these observations, the capacity of endogenous Csk and PEP molecules to physically interact in T cells was evaluated (Figure 5). The antigen-specific mouse T cell line BI-141 was lysed in NP-40-containing buffer and the presence of PEP in various immunoprecipitates was determined by immunoblotting with a rabbit antiserum generated against the catalytic domain of PEP (Figure 5A). This study showed that appreciable quantities of PEP were immunoprecipitated with $p50^{csk}$ in BI-141 T-cells (lane 2). Because the anti-PEP antibodies used in these experiments recovered at least 90% of all PEP molecules with a single immunoprecipitation (data not shown), it could be determined that 25–50% of PEP polypeptides was associated with $p50^{csk}$ in these cells (lane 2). In contrast, no PEP was detected in immunoprecipitates of $p56^{lck}$ (lane 3) or $p59^{fynT}$ (lane 4), two Src-related enzymes abundantly expressed in T cells (reviewed in Chow and Veillette, 1995), or in immunoprecipitates obtained with normal rabbit serum

(data not shown; Figure 6B, lane 3). We also tested the ability to detect $p50^{csk}$ in anti-PEP immunoprecipitates (Figure 5B). An anti-Csk immunoblot of PEP immunoprecipitates revealed that ~5% of $p50^{csk}$ was associated with PEP in BI-141 cells (lane 2). In comparison, no Csk was found in immunoprecipitates generated with normal rabbit serum (lane 3).

The association of Csk and PEP was also evaluated in *ex vivo* mouse thymocytes (Figure 6A). Once again, ~25% of PEP polypeptides was found in anti-$p50^{csk}$ immunoprecipitates (lane 2). However, no PEP was present in immunoprecipitates of Chk (lane 3), Lck (lane 4) or FynT (lane 5). Finally, the interaction between Csk and PEP was examined in other hemopoietic cells (Figure 6B). As was the case for BI-141 T-cells (lane 2), PEP was complexed with Csk in both WEHI-231 B cells (lane 5) and the RAW 264.7 macrophage cell line (lane 8). By quantitating these results, it was estimated that ~50% and ~35% of PEP was bound to $p50^{csk}$ in WEHI-231 and RAW 264.7 respectively.

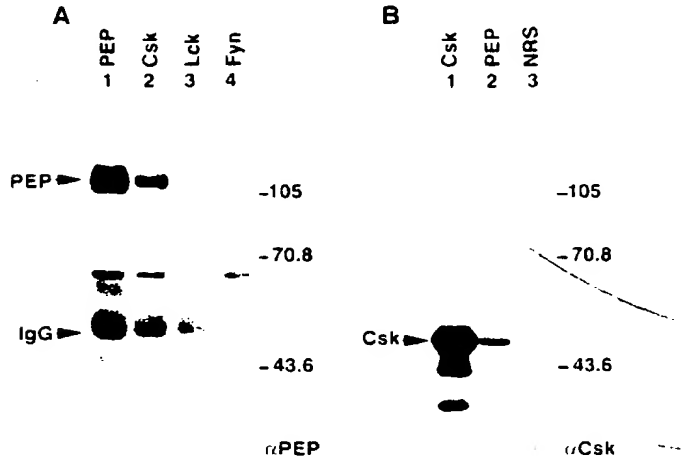


**Fig. 5.** Detection of Csk–PEP complexes in an antigen-specific T cell line. BI-141 T cells were lysed in NP-40-containing buffer and various polypeptides were immunoprecipitated from 1 mg cellular protein, using the indicated antibodies. The presence of PEP or $p50^{csk}$ in these immune complexes was determined by immunoblotting with a polyclonal antiserum directed against the catalytic domain of PEP (**A**) or against $p50^{csk}$ (**B**) respectively. The positions of PEP, the heavy chain of IgG and Csk are indicated on the left. Migration of prestained molecular weight markers is shown on the right in kDa. Exposure times: (A) 24 h; (B) 20 h.

### *Localization of PEP in Cos-1 cells and BI-141 T-cells*

An earlier report showed that PEP localized to the nucleus in HeLa cells when transiently overexpressed using the vaccinia virus system (Flores *et al.*, 1994). This observation was troubling, since Csk does not accumulate in the nucleus, but rather is present in the cytosol and, to a lesser degree, in association with cellular membranes (reviewed in Chow and Veillette, 1995). Hence, we wished to determine whether PEP was also present in the nucleus of other cells, including hemopoietic cells. To achieve this goal, the cellular distribution of PEP was first determined by immunofluorescence of transiently transfected Cos-1 cells. Cells were transfected by electroporation with the *myc–pep* cDNA, and immunofluorescence analysis was performed 48 h later, using anti-Myc mAb 9E10 and fluorescein-labeled goat anti-mouse IgG (Figure 7). While no fluorescence was noted in Cos-1 cells transfected with vector alone (Figure 7B), cells transfected with the *myc–pep* cDNA exhibited an accumulation of immunofluorescence in the cytoplasm (Figure 7A). Notably, no significant amount of PEP was observed over the nucleus. In addition, the localization of PEP in BI-141 T cells was ascertained by cell fractionation (data not shown). These experiments showed that most of PEP in BI-141 cells was present in the particulate fraction, which contains various cellular membranes but largely excludes nuclei. Hence, in combination, these results indicated that PEP was mostly localized outside the nucleus of Cos-1 cells and BI-141 T-cells.

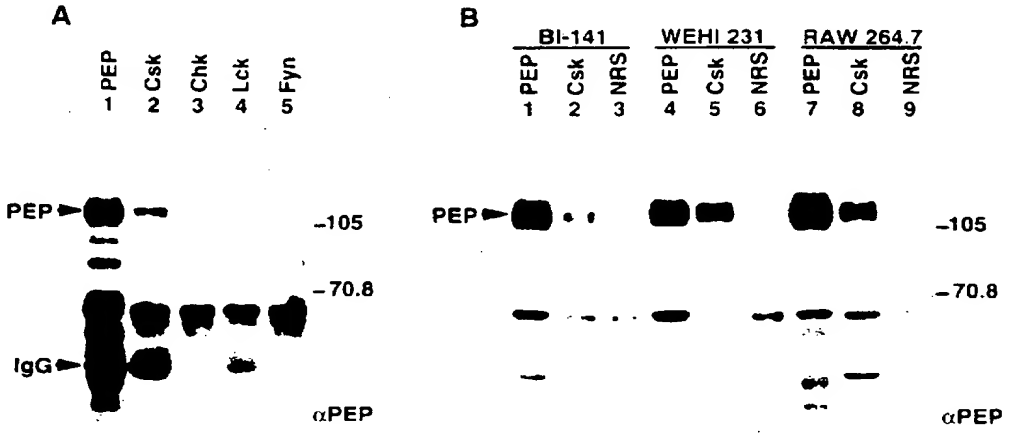


**Fig. 6.** Detection of Csk–PEP complexes in mouse thymocytes and other hemopoietic cells. (**A**) Mouse thymocytes were lysed as described in the legend to Figure 5 and lysates were subjected to immunoprecipitation with the indicated antibodies. The presence of PEP in these immunoprecipitates was determined by immunoblotting with anti-PEP antibodies. The positions of PEP and the heavy chain of IgG are shown on the left, whereas those of prestained molecular mass markers are indicated on the right. The immunoreactive product seen at ~70 kDa in all lanes is a non-specific cross-reactive protein of undefined nature. It was also detected in immunoprecipitates obtained with normal rabbit serum (Figure 6B; data not shown). Exposure time 18 h. (**B**) BI-141 T cells, WEHI-231 B cells and RAW 264.7 macrophages were processed as for (A) and subjected to immunoprecipitation with anti-PEP, anti-Csk or normal rabbit serum, followed by immunoblotting with anti-PEP antibodies. The positions of PEP and the heavy chain of IgG are shown on the left, whereas those of prestained molecular mass markers are indicated on the right. The immunoreactive product seen at ~70 kDa in all lanes is a non-specific cross-reactive protein of undefined nature. Exposure time 14 h.

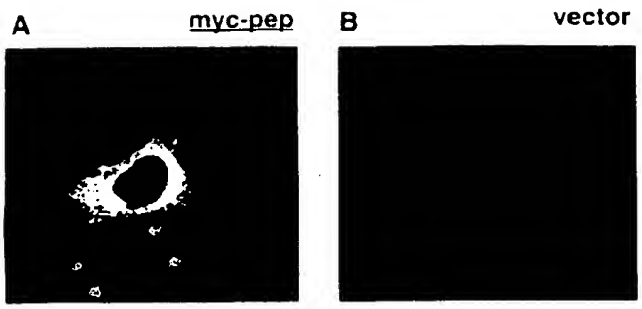


Fig. 7. Localization of PEP in transiently transfected Cos-1 cells by immunofluorescence. Immunofluorescence was studied on transiently transfected Cos-1 cells, using anti-Myc mAb 9E10. Magnification 400×. (**A**) Cells transfected with *myc-pep* cDNA. (**B**) Cells transfected with vector (pXM139) alone.

## Discussion

We have attempted to elucidate the functions provided by the SH3 and SH2 domains of Csk. Using the yeast two-hybrid system, clones encoding potential Csk-associated proteins were isolated from a mouse splenocyte cDNA library. All clones analyzed were found to be identical and to code for the C-terminal portion of PEP, a non-receptor PTP expressed in hemopoietic cells (Matthews *et al.*, 1992). The association between $p50^{csk}$ and PEP was documented in transiently transfected Cos-1 cells and in a variety of hemopoietic cells, including T cells, B cells and macrophages. Quantitative analyses showed that 25–50% of PEP and ~5% of Csk participated in Csk–PEP complexes in hemopoietic cells.

Experiments conducted in yeast and Cos-1 cells or using bacterially produced proteins showed that the Csk–PEP interaction involved the SH3 sequence of $p50^{csk}$ and a proline-rich region in the C-terminal portion of PEP. While the results obtained with these various systems generally agreed, a discrepancy was noted regarding the proline-rich motif mediating the interaction. In *in vitro* binding assays, the proline-rich region P1 (PPPLPERTP), but not P2 (PPPPLPERT), was capable of binding the SH3 region of Csk. In keeping with this finding, deletion of P1, but not of P2, abrogated the ability of full-length PEP to associate with Csk in Cos-1 cells. However, in the yeast two-hybrid system, both P1 and P2 appeared capable of mediating an association with $p50^{csk}$ (although P1 seemed more efficient). We feel that these differences likely reflect the lower stringency of the yeast system for protein–protein interactions. Consequent on the high levels of protein expression achieved in yeast, associations of lower affinity are probably permitted. Alternatively, it is possible that the ability of P2 to interact with the Csk SH3 domain was masked by determinants present in the full-length PEP protein, but not in the C-terminal fragment of PEP. This possibility is somewhat unlikely though, since a trpE fusion protein containing only 26 amino acids from PEP including the P2 motif was also inefficient at interacting with the SH3 sequence of $p50^{csk}$ *in vitro*.

The P1 sequence of PEP represents the first known ligand for the Csk SH3 domain. As it fits the consensus XPPLPXR, P1 presumably represents a class II SH3 domain ligand (reviewed in Cohen *et al.*, 1995). Interestingly, the observed inability of trpE–P2 to interact with the Csk SH3 domain *in vitro* implied that the sequence PPPLPERT is not sufficient to allow high affinity binding and that adjacent amino acids are important for the interaction. This idea is also in keeping with the recent report by Feng *et al.* (1995), highlighting the significance of residues outside the proline-rich core for high affinity SH3 domain–ligand interactions. Future studies are clearly required to examine whether this phenomenon underlies the differential ability of P1 and P2 to associate with the Csk SH3 domain.

Various lines of evidence demonstrate that the interaction between Csk and PEP is very specific. First, the proline-rich region P1 of PEP failed to bind any of the other SH3 domains tested. These included the SH3 region of Chk, the other member of the Csk family expressed in hemopoietic cells (reviewed in Chow and Veillette, 1995), and the SH3 sequences of Src-related enzymes $p56^{lck}$, $p59^{fyn}$ and $p60^{src}$. The inability of PEP to associate with Chk could also be documented in thymocytes, Cos-1 cells and yeast cells (this report; our unpublished data). In addition, a further indication of the specificity of the Csk–PEP interaction is lent by previous studies documenting the inability of the Csk SH3 sequence to associate with ligands such as dynamin, 3BP-1, Sos, c-Cbl and Sam68 (Gout *et al.*, 1993; Bowtell and Langdon, 1995; our unpublished data). Finally, we have examined the ability of PTP-PEST, a ubiquitous PTP structurally related to PEP (Yang *et al.*, 1993; Charest *et al.*, 1995), to associate with Csk (our unpublished data). Our preliminary results indicated that, even though PTP-PEST also carries the proline-rich motif PPPLPERTP in its C-terminal tail, it failed to bind Csk in transiently transfected Cos-1 cells. Perhaps this difference relates to the fact that the amino acid sequence surrounding the proline-rich region in PTP-PEST diverges from that of PEP. Alternatively, it may indicate that the conformation or cellular localization of PTP-PEST does not favor its association with $p50^{csk}$.

We were intrigued by an earlier report showing that PEP accumulated in the nucleus of HeLa cells when expressed using the vaccinia virus system (Flores *et al.*, 1994). However, through immunofluorescence and cell fractionation studies, we determined that PEP was mostly located outside the nucleus of Cos-1 and BI-141 T cells. While the exact localization of PEP in these cells remains to be determined, our data strikingly contrast with those obtained by Flores *et al.* (1994). These various findings can be reconciled by suggesting that the cellular localization of PEP may vary depending on the cell type. Alternatively, it is possible that the massive and rapid overexpression of PEP achieved with the vaccinia virus system led to artificial targeting of PEP to the nucleus (Flores *et al.*, 1994). Once the exact cellular locale of PEP is known, it will be interesting to examine whether a specific pool of Csk molecules is involved in interacting with PEP. PEP may predominantly associate with the population of $p50^{csk}$ molecules (~10%) detected in the particulate fraction (Cloutier *et al.*, 1995).

Given the specificity and relatively high stoichiometry of the Csk–PEP interaction, it is likely that PEP plays a significant role in either the regulation or the function of Csk in T cells and other hemopoietic cells. A defect in PEP binding may at least partially explain the inability of ΔSH3 Csk to inhibit antigen receptor-mediated T cell activation (Cloutier *et al.*, 1995). Moreover, it may con-

tribute to the demonstrated inefficiency of the Csk-related enzyme Chk to repress antigen receptor-triggered signals in BI-141 T cells (L.M.L.Chow and A.Veillette, unpublished). The physical interaction of Csk and PEP could have several consequences. First, either enzyme could regulate the function of the other, through changes in phosphorylation or conformation. This model appears somewhat unlikely, however, as we failed to document Csk-mediated phosphorylation of either wild-type or catalytically inactive PEP *in vitro* (our unpublished data). Moreover, Csk is not detectably tyrosine-phosphorylated *in vivo*, even in the absence of PEP (our unpublished data). Second, PEP could restrict the function of Csk in T cells by preventing phosphorylation of a subset of Csk substrates. This possibilily also appears improbable, as the pattern of tyrosine protein phosphorylation induced by Csk in T cells is not modified by deletion of its SH3 domain (Cloutier *et al.*, 1995). Third, Csk and PEP could cooperate to regulate common intracellular targets. For example, while Csk inhibits Src family kinases by phosphorylating their C-terminal tyrosine, PEP may have a similar effect by dephosphorylating their positive regulatory site. Alternatively, PEP may amplify the impact of Csk by dephosphorylating some of the targets of Src family kinases. Clearly, the resolution of these hypotheses may yield important information regarding the regulation and function of Csk in T cells and other hemopoietic cells.

In summary, we have found that the inhibitory TPK $p50^{csk}$ interacts with the non-receptor PTP PEP in hemopoietic cells. This association involves the SH3 domain of $p50^{csk}$, and a proline-rich motif (PPPLPERTP) in the non-catalytic C-terminal region of PEP. These findings constitute the first characterization of a ligand for the Csk SH3 domain. Moreover, they suggest that PEP is involved in either regulating or effecting the function of Csk in hemopoietic cells.

## Materials and methods

### Cells

The antigen-specific mouse T cell line BI-141 and the macrophage cell line RAW 264.7 have been described elsewhere (Koren *et al.*, 1975; Reske-Kunz *et al.*, 1985). The WEHI-231 B cell line was purchased from the American Type Culture Collection (Rockville, MD). All hemopoietic cells were propagated in RPMI medium supplemented with 10% fetal calf serum (FCS) and antibiotics. Cos-1 cells were grown in α-modified essential medium (MEM) containing 10% FCS and antibiotics. Thymocytes were obtained from 4–6-week-old Balb/c mice.

### Antibodies

Anti-Csk antibodies were produced in rabbits using a synthetic peptide corresponding to the last 13 amino acids of rat Csk (Nada *et al.*, 1991), coupled to keyhole limpet hemocyanin. Similar anti-Csk antibodies were also obtained from Drs Brian Howell and Jon Cooper (Fred Hutchinson Cancer Center, Seattle, WA; Howell and Cooper, 1994). Other anti-Csk antisera directed against the kinase region of $p50^{csk}$, as well as antisera reacting against the unique domain of $p56^{lck}$ or $p59^{fyn}$ or against the C-terminal tail and kinase domain of Chk have been reported elsewhere (Veillette *et al.*, 1988; Chow *et al.*, 1993, 1994b; Davidson *et al.*, 1994). Anti-trpE mAbs were purchased from Oncogene Science (Cambridge, MA). Mouse anti-Myc mAb 9E10 and anti-HA mAb 12CA5 have been previously described (Evan *et al.*, 1985; Charest *et al.*, 1995) and were used as purified antibodies. Anti-PEP antibodies were generated in rabbits using trpE fusion proteins containing either amino acids 303–524 (antiserum 79) or amino acids 1–269 (antiserum 87) of PEP. These two sequences correspond to the N-terminal portion of the non-catalytic domain of PEP and the phosphatase domain of PEP respectively (Matthews *et al.*, 1992).

### cDNAs and mutants

The cDNA encoding rat $p50^{csk}$ was provided by Dr Masato Okada (Osaka, Japan; Nada *et al.*, 1991). A full-length mouse *pep* cDNA was cloned from a fetal thymus library (provided by Dr Louis Matis, Alexion Pharmaceuticals, New Haven, CT), using the *pep* cDNA clone obtained with the yeast two-hybrid system as a probe. The entire *pep* cDNA was resequenced to ensure that no mutation was present (data not shown). cDNAs coding for Csk mutants lacking the SH3 (ΔSH3 Csk) or SH2 (ΔSH2 Csk) domain or devoid of catalytic activity [Lys222→Arg222 (R222 Csk)] have been reported elsewhere (Cloutier *et al.*, 1995). cDNAs encoding Csk polypeptides bearing three copies of an HA tag at the C-terminus (Csk–HA) or PEP molecules bearing a Myc epitope at the N-terminus (Myc–PEP) were produced by PCR. PEP mutants lacking the first (PPPLPERTP; ΔP1) or second (PPPPLPERT; ΔP2) proline-rich motif were generated by overlap extension PCR. All mutated cDNAs were resequenced to verify that they lacked unwanted mutations (data not shown).

### Yeast two-hybrid system

The full-length rat *csk* cDNA was cloned in-frame in the yeast expression vector pBTM116, which contains the lexA DNA binding domain. Then it was introduced into the yeast strain L40 by lithium acetate transformation. As pBTM116 also contains the tryptophan (*trp*) gene, transformed yeast cells were selected for growth in tryptophan-deficient medium. Colonies expressing the lexA–Csk fusion were identified by immunoblotting with anti-Csk antibodies (data not shown). A mouse splenocyte library fused to the VP16 transactivation domain (provided by Dr Andrey Shaw, St Louis, MO) was introduced into lexA–Csk-expressing L40 cells. Since the library vector pVP16 carries the leucine (*leu*) gene, it conferred the capacity to grow in leucine-deficient medium. In addition, binding of library-encoded products to the 'bait' (Csk) allowed transactivation of the lexA DNA binding domain and expression of the *his* and *lacZ* genes. These two genes allowed growth in histidine-deficient medium and green staining in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (see below) respectively. A total of $\sim 20 \times 10^6$ library transformants were screened in this manner. Approximately 120 of these colonies were positive for both *his* and *lacZ*. Ninety five were randomly selected and subjected to further study.

To exclude 'false positives', the Csk 'bait' was eliminated by prolonged growth in tryptophan-containing medium. As a result, 95 colonies lost their β-galactosidase activity (data not shown). These potential 'true positives' were further tested by introduction of other lexA DNA binding domain fusions, including lexA alone, lexA–lamin and lexA–Raf (kindly provided by Dr A. Shaw). In contrast to lexA–Csk, these various fusions were unable to induce β-galactosidase activity in 90 of the 95 clones (data not shown). Plasmids were recovered from these colonies by DNA extraction and transferred to *E.coli* in order to obtain larger amounts of DNA. Inserts were analyzed by restriction digestion and/or sequencing (data not shown). All 90 plasmids were found to contain an identical insert, corresponding to the non-catalytic C-terminal portion of PEP (Figure 1).

To determine the structural requirements in Csk for interaction with PEP, yeast cells expressing the C-terminal segment of PEP were transformed with plasmids encoding lexA–Csk variants bearing a deletion in either the SH3 (ΔSH3 Csk) or SH2 (ΔSH2 Csk) domain or carrying a point mutation at the ATP binding site (R222 Csk). Similarly, to address the role of the SH3 domain, cells were transformed with a lexA fusion encompassing only the SH3 region of $p50^{csk}$. After confirming expression of these various fusions by immunoblotting with anti-Csk antibodies (data not shown), their ability to associate with PEP was ascertained by assaying for β-galactosidase activity (see below).

To map the PEP sequences necessary for binding to Csk, various N-terminal and C-terminal truncations were created in the C-terminal portion of PEP (C-PEP) by PCR, as depicted in Figure 2. The resulting cDNA fragments were cloned in-frame in pVP16. After introduction of these plasmids in Csk-containing yeast, assays of β-galactosidase activity were conducted as outlined below.

### β-Galactosidase assays

To measure expression of the *lacZ* gene in yeast, colonies were blotted onto a nitrocellulose filter and subjected to flash freezing in liquid nitrogen. The filter was allowed to thaw at room temperature and then laid on a Whatman filter paper, pre-humidified with a solution containing 60 mM $Na_2HPO_4$, 40 mM $NaH_2PO_4$, 10 mM KCl, 1 mM $MgSO_4$ and 1 mM X-Gal. Filters were incubated at 30°C for the indicated period of time and green staining of colonies was assessed visually.

### *Bacterial fusion proteins*

To study the interaction of PEP with the SH3 region of Csk *in vitro*, various trpE fusion proteins were produced. The necessary DNA fragments from *pep* were amplified by PCR and cloned in-frame in the appropriate pATH vector. The resulting trpE fusion proteins encompassed amino acids 303–524 (N-terminus), 605–629 (P1), 680–705 (P2), 605–705 (P1 + P2) or 714–802 (P3 + P4) of PEP (Figure 3). All plasmids were verified by sequencing (data not shown). Plasmids encoding GST fusion proteins bearing the SH3 domain of Csk, Chk, Lck or Fyn were generated by PCR. Plasmids encoding other GST–SH3 domain fusion proteins were generously provided by Dr Tony Pawson (Mount Sinai Hospital, Toronto, Ontario, Canada). Production and purification of GST fusion proteins were as described elsewhere (Peri *et al.*, 1993).

### *In vitro binding assays*

Bacterial lysates (50 µg) containing the appropriate trpE fusion proteins were produced as explained previously (Veillette *et al.*, 1992). They were then incubated with ~1 µg of various GST fusion proteins immobilized on agarose–glutathione beads (Peri *et al.*, 1993). After several washes, bound proteins were resolved by SDS–PAGE and detected by immunoblotting with anti-trpE antibodies. The presence of comparable quantities of the various GST fusion proteins was confirmed by Coomassie blue staining of parallel samples resolved in SDS–PAGE gels (data not shown).

### *Transfections*

cDNAs were individually inserted in the multiple cloning site of pXM139, a vector which bears the SV40 origin of replication. Cos-1 cells were transfected by the DEAE–dextran method, using a fixed total amount of DNA (8 µg) (Rodriguez and Park, 1993). After 12 h, cells were incubated with chloroquine (60 µg/ml) for 5 h. Following an additional growth period of 48 h, cells were processed for immunoprecipitation and immunoblotting.

### *Immunoprecipitations and immunoblots*

After washing in phosphate-buffered saline (PBS), cells were lysed in TNE buffer [50 mM Tris, pH 8.0, 1% Nonidet P-40 (NP-40), 2 mM EDTA] supplemented with 10 µg/ml each of the protease inhibitors leupeptin, aprotinin, *N*-tosyl-L-phenylalanine chloromethyl ketone, *N*-*p*-tosyl-L-lysine chloromethyl ketone and phenylmethylsulfonyl fluoride, as well as the phosphatase inhibitors sodium fluoride (50 mM) and sodium orthovanadate (1 mM). Polypeptides were recovered by immunoprecipitation from equivalent amounts of total cellular proteins using the indicated antibodies. Immune complexes were collected with *Staphylococcus aureus* protein A (Pansorbin; Calbiochem), coupled, if indicated, to rabbit anti-mouse IgG. Immunoprecipitates were washed three times with TNE buffer containing 1 mM sodium orthovanadate. Proteins were then eluted in sample buffer, boiled, electrophoresed in 8% SDS–PAGE gels and transferred onto Immobilon membranes (Millipore) for immunoblotting. Immunoblots were performed according to a previously described protocol (Veillette *et al.*, 1988). After incubation with $^{125}$I-labeled protein A (Amersham) or $^{125}$I-labeled goat anti-mouse IgG (ICN), immunoreactive products were detected by autoradiography and quantitated with a Phosphorimager (BAS 2000; Fuji).

### *Immunofluorescence*

Cos-1 cells were transfected by electroporation with pXM139 alone or pXM139 bearing a *myc–pep* cDNA. Then cells were seeded onto Lab-Tek Chamber Slides (Nunc-Inc., Naperville, IL) and grown at 37°C for 48 h. They were subsequently washed in PBS, fixed in PBS containing 4% paraformaldehyde and permeabilized with saponin (0.1%). After blocking non-specific sites with 3% bovine serum albumin, cells were incubated for 1 h at room temperature with anti-Myc mAb 9E10. Cells were extensively washed and exposed for 1 h in the dark to fluorescein isothiocyanate-conjugated F(ab')$_2$ fragments of goat anti-mouse IgG (Organon Teknika, West Chester, PA). Following several additional washes in PBS containing 0.1% saponin, immunoreactivity was detected by fluorescence microscopy.

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